Understanding human immunology through the study of primary immune deficiency disorders

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CHAPTER 3

NRAS MUTATION CAUSES A HUMAN AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

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ABSTRACT
The p21 RAS subfamily of small GTPases, including KRAS, HRAS, and NRAS, regulates cell proliferation, cytoskeletal organization and other signaling networks, and is the most frequent target of activating mutations in cancer. Activating germline mutations of KRAS and HRAS cause severe developmental abnormalities leading to Noonan, cardio-facial-cutaneous and Costello syndrome, and somatic events are associated with many forms of cancer. Autoimmune lymphoproliferative syndrome (ALPS) is the most common genetic disease of lymphocyte apoptosis and causes autoimmunity as well as excessive lymphocyte accumulation, particularly of CD4-, CD8- ab T cells. Mutations in ALPS typically affect CD95 (Fas/APO-1)-mediated apoptosis, one of the extrinsic death pathways involving tumor necrosis factor receptor (TNFR) superfamily proteins, but certain ALPS individuals have no such mutations. We show here that the salient features of ALPS as well as a predisposition to hematological malignancies can be caused by a heterozygous somatic Gly13Asp activating mutation of the NRAS oncogene that does not impair CD95-mediated apoptosis. The increase in active, GTP-bound NRAS augments RAF/MEK/ERK signaling which markedly decreases the pro-apoptotic protein BIM and attenuates intrinsic, nonreceptor-mediated mitochondrial apoptosis. Thus, somatic activating mutations in NRAS can be associated with a non-tumoral phenotype in humans. Our observations on the effects of NRAS activation indicate that RAS-inactivating drugs, such as farnesyl-transferase inhibitors (FTIs) should be examined in human autoimmune and lymphocyte homeostasis disorders.

INTRODUCTION
The RAS genes (NRAS, KRAS, and HRAS) encode 21-kDa proteins that are members of the superfamily of small GTP-binding proteins, which have diverse intracellular signaling functions including control of cell proliferation, growth, and apoptosis. Somatic activating mutations in RAS are present in up to 30% of all human cancers. Germline RAS pathway mutations have only recently been described as causing the related Costello (HRAS), Noonan (PTPN11, KRAS, SOS1), and cardiofaciocutaneous syndromes (KRAS, BRAF, MEK1, and MEK)3-7. Individuals with these syndromes typically present
with severe developmental anomalies in various combinations of facial abnormalities, heart defects, short stature, skin and genital abnormalities, and mental retardation\(^8\). Defects in the immune system have not been reported. Patients with Costello and Noonan syndromes have an increased propensity to solid and hematopoietic tumors, respectively \(^3,8\). Germline mutations in \textit{NRAS} have not yet been described.

The autoimmune lymphoproliferative syndrome (ALPS) (OMIM 601859/603909) is the most common genetic disorder of lymphocyte apoptosis and is characterized by chronic accumulation of nonmalignant lymphocytes, defective lymphocyte apoptosis, and an increased risk for the development of hematological malignancies\(^9\). A signature of the disease is the accumulation of \(\alpha\beta\) T cells lacking the CD4 and CD8 coreceptors that are termed “double-negative” T cells (DNTs: CD4\(^-\) CD8\(^-\) TCR\(\alpha\beta\)\(^+\) cells). These cells bear no known relationship to thymic DNTs, a stage that occurs before \(\alpha\beta\) TCR gene rearrangements in ontogeny\(^10\). According to genotype, ALPS can be classified as types Ia, Ib, and II, which are due to germline mutations in CD95 (\textit{TNFRSF6}), CD95 ligand (\textit{TNFSF6}), and caspase 10 (\textit{CASP10}), respectively\(^11-15\). Additionally, somatic mutations of CD95 in \(\alpha\beta\) DNTs can also cause ALPS of type Im (mosaic)\(^16\). All of these mutations impair extrinsic, Fas receptor-mediated apoptosis. An enigma has been the ALPS individuals who have no defects in CD95 pathway apoptosis (some ALPS type III patients)\(^17\). This group encompasses a large number of individuals and is probably genetically heterogeneous. In an attempt to unveil new genetic defects, we investigated alternative apoptosis pathways in ALPS type III and identified one ALPS patient with a unique defect in cytokine withdrawal-induced apoptosis due to an activating somatic NRAS mutation affecting hematopoietic cells.

**METHODS**

\textit{Cells and treatments}

Patients were studied under an NIH IRB approved ALPS research protocol after obtaining informed consent. Peripheral blood lymphocytes (PBL) were isolated by Ficoll-Hypaque gradient centrifugation, and cultured in RPMI-1640 media (supplemented with 2\(\mu\)M L-glutamine, 10 mM HEPES, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, 10\% fetal bovine serum). PBL were activated with 1 \(\mu\)g/ml of anti-CD3 (OKT3, Ortho
Biotech, Bridgewater, NJ) and 25 IU/ml of recombinant human IL-2 (Roche Applied Science, Indianapolis, IN) for 3 days. The activated cells were washed twice with PBS and cultured for at least 3 additional days in complete media supplemented with 100 IU/ml of IL-2. Cells were used for experiments between the 6th and 21st days in culture. For cytokine withdrawal assays, IL-2-containing media was changed 24 h prior to experiments. The IL-2-treated cells were then washed 3 times with PBS and re-suspended at 1x10^6 cells/ml in complete media without IL-2 and cultured for different periods of time. For other apoptosis assays, activated lymphocytes were treated with staurosporine (Calbiochem, EMD Biosciences San Diego, CA), an agonistic anti-Fas antibody Apo1.3 (Alexis, San Diego, CA) orγ-irradiated. Apoptosis was determined by measuring plasma membrane integrity and loss of the mitochondrial transmembrane potential, through simultaneous staining with 50 ng/ml of propidium iodide (PI), and 40 nM of 3, 3'-dihexyloxacarbocyanine iodide (DiOC6) (Calbiochem, EMD Biosciences) for 15 min at 37°C. Live cells (PI negative and DiOC6 positive) were collected by flow cytometry using a constant time acquisition. The % of cell loss was calculated according to the formula: \((\text{number of live cells before treatment} - \text{number of live cells after treatment}) / \text{number of live cells before treatment}) \times 100\). “Cell survival” was calculated as: 100 - % cell loss. Resting T cells were purified by negative selection using CD4 T cell separation kit II (Miltenyi Biotec, Auburn, CA). Resting B cells and monocytes were isolated by positive selection using PE-conjugated anti-CD19 or anti-CD14 antibodies (BD) and then anti-PE magnetic beads (Miltenyi Biotec). The purity of separated cell populations was assessed by flow cytometry and it varied from 90 to 95% for T cells and 75 to 85% for B cells and monocytes. Chemical inhibitors PD98059, U0126, LY294002 and FTI-277 were from Calbiochem.

**Immunoblotting**

Stimuli were terminated by addition of ice-cold PBS and cell pellets were lysed immediately with RIPA buffer (10 mM Tris-HCl pH 7.0, 150 mM NaCl, 0.1% NP40, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate) containing 1x protease inhibitor cocktail (Complete Mini, Roche) and nuclease (Benzonase®, Novagen, EMD Biosciences). Samples were incubated on ice for 30 min and spun at 12,000xg for 5 min
at 4-8°C. The lysates were utilized immediately or stored at –80°C. Protein concentration was determined by the Bradford method (Micro BCA, Pierce, Rockford, IL) and 20 µg of protein was separated by polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA). Proteins were transferred onto a nitrocellulose membrane, and incubated with blocking buffer (0.1% Tween 20, 5% non-fat dry milk in PBS) at room temperature (RT) for 1h. Membranes were then incubated at 4°C overnight with primary antibodies diluted in blocking buffer under constant agitation. Membranes were washed 3 times with 5 min intervals using PBS-T (0.1% Tween 20 in PBS), and incubated with HRP-conjugated secondary antibodies at RT for 1h. Membranes were then washed 3 times, incubated with chemiluminescent reagents and exposed to film. Antibodies and sources are: anti-BIM, anti-BAK (Stressgen, Ann Arbor, MI); anti-BCL-2, anti-p27kip1, anti-MCL-1, anti-β-actin, anti-BAX, anti-cytochrome c (clone 7H8.2C12), anti-cytochrome c oxidase IV subunit II, anti-BCL-XL (BD Biosciences, San Jose, CA); anti-PUMA (Axxora, San Diego, CA); anti-AIF, anti-N-RAS (Clone F155) (Santa Cruz, Santa Cruz, CA).

Quantitative Real-Time PCR
Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. To minimize genomic DNA contamination and amplification, we performed an on-column DNase I (Qiagen) treatment, and used primers spanning exon-exon boundaries and included non-reverse-transcriptase controls in all reactions. First strand complementary DNA (cDNA) synthesis was accomplished by using Superscript III (Invitrogen), and the cDNA was used immediately used for quantitative polymerase chain reaction (qPCR) or stored at -20°C. qPCR was done on a 7700 ABI PRISM instrument with all the probes and primers purchased from the same company (Applied Biosystems). In brief, 1/20th (1 µL) per well of the cDNA reaction was aliquoted in triplicate in 96-well optical plates, and a mixture was added containing TaqMan(R) universal PCR master mix and the final concentrations of 0.9 µM of specific primers and 0.25 µM of the fluorescent probe, for a final volume of 50 µL. Thermal cycle conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Relative gene expression was calculated using the 2-ΔΔCt method after normalization using a housekeeping gene (18S rRNA), as described18. The
values were again normalized against one normal controls in each experiment, thus to calculate the fold changes in contrast to the normal. Controls for exogenous contamination (no template) were included in all qPCR experiments. Additionally, the efficiency of the primer/probe pair used for a targeting gene was compared to that of the housekeeping gene for each new set of primer and probes.

Subcellular fractionation
Cytosolic extracts were prepared by a selective digitonin-based plasma membrane permeabilization technique. An amount of 5 x 10^6 cells was washed twice in PBS and incubated on ice with extraction buffer (20 µg/ml digitonin, 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, pH 7.5, 1 x protease inhibitor cocktail) for 5 min. Permeabilization was monitored by trypan blue incorporation until at least 80% of the cells became trypan-blue positive. Cells were then spun at 300x g for 10 min at 4°C and the supernatant was collected (cytosolic fraction). The pellet (membrane fraction) was lysed using 3% SDS-containing RIPA buffer at room temperature for 30 min. The purity of cytosolic fraction was evaluated by blotting for mitochondrial cytochrome oxidase II or Ox P4.

Small-interfering RNA and transient transfections
Normal human PBL were separated and activated as described above. Activated PBL were transfected with either a small interfering oligonucleotide RNA (siRNA) or a scrambled non-silencing control oligo (nsRNA). siRNAs were designed online using the software BLOCK-iTTM RNAi designer from Invitrogen (https://rnadesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=stealth) and synthesized by the same company. Transfection of activated T cells was carried out by electroporation using the Nucleofection system (Amaza, Koln, Germany), according to the manufacturer’s protocol. Briefly, 4 x 10^6 lymphocytes were resuspended in 100 µl of T cell nucleofector solution (Human T Cell Nucleofector kit, Amaza) containing 200 pmol of double-stranded siRNA or nsRNA and electroporated using the program T23. Assessment of knockdown efficiency and experiments were performed 3 days later by immunoblotting. The sequence of the sense oligonucleotides used to knockdown BIM
are: #1 GGAUCGCCCAAGAGUUGCGGCGUAU and #2 GGCCUAUUUCAGAGGAAUAUGUAA. For N-ras knockdown we used: #1 GCGCACUGACAAUCCAGCUAAUCCA; #2 CCAGCUAAUCCAGAACCACUUUGUA; #3 GGACAUACUGGAUACAGCUGGACAA. Scrambled oligonucleotides were used as non-silencing controls. Plasmids expressing human wild-type NRAS (kindly provided by Silvio Gutkind, NIDCR) and G13D mutant were transiently transfected into primary human PBL (kit T, Amaxa), Jurkat A3 (kit V, Amaxa) and H-9 (BTX) by electroporation.

**Immunofluorescence**

Cells were fixed with 4% paraformaldehyde in PBS at 4 °C for 20 min, and cytospun onto slides. Cells were then permeabilized with 0.05% Triton X-100 (or CHAPS 0.001%, for BAX staining) at RT for 5 min, washed and blocked with 10% fetal calf serum in PBS for 30 min. Samples were then incubated with primary antibodies diluted in 0.5% BSA at RT for 45 min. After 3 washes with PBS, cells were incubated with fluorochrome-conjugated secondary antibodies diluted in 0.5% BSA at RT for 45 min. After a new round of washes, nuclei were stained with 40 ng/ml Hoechst 33342 (Molecular Probes, Invitrogen, Eugene, OR). Slides were then washed in PBS and mounted with a coverslip using Fluoromount-G (Southern Biotechnology, Birmingham, AL). Images were acquired on a Leica TCS-NT/SP confocal microscope using a 63x oil immersion objective. For enumeration, a blinded observer using a conventional fluorescence microscope counted at least 200 cells per sample. Antibodies used: anti-cytochrome c (6H2.B4, BD Pharmingen), anti-HSP60 (E-1, Santa Cruz), and anti-BAX (NT, Upstate, Charlottesville, VA).

**Active NRAS pull down**

Active GTP-bound NRAS was immunoprecipitated using the EZ detection Ras activation kit (Pierce), according to the manufacture’s protocol. The immunoprecipitated proteins were separated by SDS-PAGE and probed with an anti-NRAS antibody (F-155, Santa Cruz).
DNA Sequencing

DNA samples were isolated from P58 and an unrelated, healthy individual (wild-type control). Selected regions of the following loci were amplified using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ): BCL2L11 (BIM), MAPK3 (ERK1), MAPK1 (ERK2), MAPK14 (p38α), MAPK11 (p38β), MAPK8 (JNK1), MAPK9 (JNK2), MAP2K3 (M KK3), MAP2K6 (M KK6), MAP2K4 (M KK4), MAP2K7 (M KK7), DUSP1 (CL100/M KP1), DUSP2 (PAC1), DUSP4 (hVH2/M KP2), DUSP5 (B23/hVH3), DUSP6 (M KP3), DUSP7 (PYST2/M KPX), DUSP9 (M KP4), FOXO3A, FOXO1A, FOXO4, SOS1 and NRAS. The PCR products were directly sequenced using ABI Prism BigDye (v 1.1) terminators and analyzed on an ABI 3100 Sequencer (PE Applied Biosystems, Foster City, CA). Primer sequences and annealing temperatures are available upon request. The sequencing data for the patient and wild-type control was compared with Ensembl (http://www.ensembl.org) data (v. 35 - Nov2005) for each locus. All identified mutations/SNPs were confirmed by sequencing a second PCR product.

Microarray Analysis

RNA expression in lymphocytes from P58 was compared to that in 2 normal controls (NL1, NL2) at 0 and 24 hours post IL-2 withdrawal. Total RNA was extracted, reverse transcribed, biotin labeled, fragmented, and hybridized to human Affymetrix U133Plus2.0 microarrays following standard Affymetrix procedures (Affymetrix, Santa Clara, CA). After staining with streptavidin-phycoerythrin (Molecular Probes) and biotinylated anti-streptavidin antibody (Vector Laboratories), microarrays were scanned in the CCMD/CC Functional Genomics and Proteomics Facility using the Affymetrix GeneChip Scanner. Summary probe set intensities were computed from the resulting images using GCOS version 1.2 software. Results were deposited into the NIHLIMS database, then retrieved and analyzed using the MSCL Analyst's Toolbox (http://affylims.cit.nih.gov) and the JMP statistics package (SAS, Inc., Cary, NC). Briefly, signal intensities were quantile-normalized and transformed using an adaptive variance stabilizing transform, termed "S10". A two-way ANOVA on time (0, 24 hr) and patient group (NL, P58) was performed on each gene. The difference between P58 and NL was calculated along with the significance of the patient group variable. Genes
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were selected if this difference was greater than two-fold and the change among the three patient groups reached $P < 0.05$, unadjusted. This resulted in a list of 205 probe sets, corresponding to 158 unique genes and 31 probe sets without usable annotations. A hierarchical cluster analysis on the difference from mean expression for the 205 probe sets was used to construct a heatmap and dendrogram, using the six samples from the NL and P58 groups, thus highlighting groups of similarly behaving genes. This gene list was then thematically explored using Ingenuity Pathways Analysis 3.0 (Ingenuity Systems).

**RESULTS**

**Defective IL-2 Withdrawal-Induced Apoptosis in a Patient with Clinical and Laboratory Hallmarks of ALPS**

The intrinsic mitochondrial pathway of apoptosis can be triggered by developmental cues in the thymus or bone marrow, cytokine deprivation, DNA damage, or treatment with cytotoxic drugs\textsuperscript{20,21}. To screen for defects in this pathway, we exposed activated lymphocytes from individuals with salient features of ALPS (lymphadenopathy and increased $\alpha\beta$ DNTs) but normal CD95-mediated apoptosis, to inducers of intrinsic apoptosis including staurosporine, $\gamma$-radiation, and cytokine withdrawal. We identified an individual whose lymphocytes clearly resisted death induced by IL-2 withdrawal (Fig. 1A).

The affected individual [National Institutes of Health (NIH) cohort patient 58, P58] is a 49-year-old male with lifelong overexpansion of lymphocytes, and an unusual history of two malignancies: childhood leukemia and early adulthood lymphoma, both successfully treated (Table 1). Peripheral blood immunophenotyping revealed a mild but sustained elevation in $\alpha\beta$ DNT cells over several years and other findings frequently seen in ALPS, including an elevated percentage of CD5$^+$ B cells and low numbers of CD27$^+$ B cells\textsuperscript{22}. However, other features such as low CD25/HLA-DR ratio and high numbers of CD3$^-$/CD57$^+$ were not seen (Table 1). Lymph node biopsy performed elsewhere and reviewed at the NIH revealed reactive follicular hyperplasia and sinus histiocytosis, but DNT cells were not prominent. Several serum autoantibodies were detected and elevations of several T helper 2 cytokines including IL-5, -6, -8, -10, and -13 were observed (Tables 1 and data not shown). Based on the published NIH ALPS diagnostic
criteria of elevated DNT on peripheral blood, chronic lifelong nonmalignant hyperplasia and defective lymphocyte apoptosis, P58 received a provisional clinical diagnosis of ALPS, with recognition that this is not a typical clinical presentation of this disease.

Despite defective IL-2 withdrawal death, we found no abnormalities in apoptosis induced by an agonistic anti-APO-1 (CD95) antibody (Apo1.3), staurosporine, or γ-radiation (Fig. 1B–D). Moreover, activated T cells did not spontaneously proliferate or secrete cytokines, but did show persistent proliferation after IL-2 withdrawal compared with normal cells (Fig 2. and data not shown). Thus, lymphocytes from P58 demonstrated a specific defect in the intrinsic pathway of apoptosis.

**Table 1. Clinical and laboratory findings in P58**

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<th>Clinical history</th>
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<td>Lifelong lymphadenopathy and splenomegaly</td>
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<td>Marked leukocytosis (≥100,000 cels/mm³) with 40 % blasts noted on peripheral smear at 8 months of age treated with oral agents, resolution by age 4 years</td>
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<tr>
<td>Large, non-cleaved, non-Hodgkin’s B cell-lymphoma at 32 years of age</td>
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<td>Currently well at 48 years with persistent lymphadenopathy</td>
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**Laboratory findings**

Positive autoantibodies: direct antiglobulin test, ANA, ACA IgG and IgM, rheumatoid factor

| Total leukocytes (3,300-9,600/mm³) = 9,630/mm³ |
| Total lymphocytes (460-4,700/mm³) = 3,929/mm³ |
| CD3+/CD4+ (28.6-57.2%/358-1259/mm³) = 19.9 % (782/mm³) |
| CD3+/CD8+ (12.9-46.9%/194-836/mm³) = 7.8 % (306/mm³) |
| CD3+/CD4-/CD8-/TCRαβ+ (<1%/<20/mm³) = 2.4% (94/mm³) |
| CD20+ (3.7-15.5%/49-424/mm³) = 57.5 % (2259/mm³) |

* Adult reference range for each value is shown in parentheses. ANA, antinuclear antibodies; ACA, antificardiolipin antibodies
Figure 1. Defective cytokine withdrawal-induced apoptosis in P58 lymphocytes. (A–D) Activated peripheral blood mononuclear cells (PBLs) from normal volunteers (NL A and B), from a patient with an inactivating Fas mutation (ALPS 1A), and from P58 were cultured in media without IL-2 for the indicated periods of time (A); or treated for 18 h with anti-Fas (Apo1.3) antibody (B), staurosporine (C), or γ-irradiation (D) at the indicated doses. (E) (a–d) Merged views of active BAX (green) and Hoechst nuclear staining (blue) of cells from P58 and a normal control (NL) at 0 h (a and b) and 72 h after cytokine withdrawal (c and d). (e–h) Merged views of staining with the mitochondrial marker Hsp60 (green), cytochrome c (red), and Hoechst staining (blue) at 0 h (e and f) or 72 h after IL-2 withdrawal (g and h). (F) Quantitation using fluorescence microscopy of the relative proportion of cells in the experiment in E showing active BAX expression (activ. BAX), diffuse cytochrome c (diff. cyt. c), or apoptotic (apop.) nuclei. Data shown are the representative of two or three independent experiments. Shown is mean ± SD.
Figure 2. In vitro cellular proliferation and viability of P58 lymphocytes. (A) Activated lymphocytes from 4 normal donors (NL 1 to 4), an ALPS 1A patient and P58 were cultured in media containing 100 IU/ml of IL-2, and incorporation of [3H]-thymidine analyzed 18 h later. (B) Activated lymphocytes from NL1, NL2, an ALPS 1A patient and P58 were cultured as described in A and total cell numbers counted daily by flow cytometry. (C) [3H]-thymidine incorporation by activated lymphocytes from a NL and P58 that were cultured in media without IL-2 for 24h. (D) Activated lymphocytes from 3 normal donors (NL1-3), an ALPS 1A patient and P58 were cultured in media without IL-2 and cell death was measured by staining with propidium iodide (PI) and 3, 3'-dihexyloxacarbocyanine iodide (DiOC6) followed by flow cytometric analysis.

During cytokine withdrawal, the proapoptotic B cell lymphoma 2 (BCL-2) family members BAX and BAK are activated and oligomerize at mitochondrial surfaces, causing permeabilization of the outer membrane, which releases cytochrome c and triggers apoptosis. After IL-2 withdrawal, we found that P58 cells were markedly defective in BAX activation and mitochondrial cytochrome c release, correlating with a
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decreased percentage of apoptotic nuclei compared with control cells after cytokine withdrawal, but not staurosporine treatment, clearly indicating a selective mitochondrial apoptosis defect (Fig. 1E and F, and Fig. 3A and B)

**Figure 3. Analysis of cytosolic release of cytochrome c and AIF by subcellular fractionation.** Immunoblotting of cytochrome c (Cyt C), apoptosis inducing factor (AIF), Cox IV, and β-actin in the cytosols of activated T lymphocytes from a NL, an ALPS 1A patient, and P58 that have been deprived of IL-2 for 72 h (A) or treated with 500 nM of staurosporine (STS) for 2 h (B). The degree of purity of the cytosolic fractions is demonstrated by probing with the mitochondrial proteins CoxIV (A) or OxP4 (B), CW, cytokine withdrawal.

**Diminished BCL-2-Interacting Mediator of Cell Death (BIM) Levels in P58**
Critical initiators of intrinsic apoptosis that act upstream of BAX and BAK are the BCL-2 homology 3 (BH3) subclass of the BCL-2 family. Knockout mice lacking BIM, a BH3-only protein, manifest immune abnormalities including lymphocyte accumulation, autoimmunity, and various apoptosis defects, especially cytokine withdrawal-induced cell death. We therefore measured BIM levels and found markedly reduced basal and induced levels after IL-2 withdrawal from activated T cells in P58 compared with normal or ALPS type 1A controls (Fig. 4A). This defect was specific to BIM because other proapoptotic (PUMA, BAX) and antiapoptotic (BCL-2, BCL-XL, MCL-1) BCL-2 family members were unaffected (Fig. 4A). We also observed a constitutive BIM deficiency in resting peripheral blood lymphocytes (PBLs) as well as purified T and B cells from P58 (Fig. 4B).
In an additional effort to demonstrate the importance of BIM for mitochondrial apoptosis in human cells, we used small interfering RNA (siRNA) to silence BIM expression in otherwise normal human lymphocytes. BIM suppression caused resistance to IL-2 withdrawal, but not Fas- or staurosporine-induced apoptosis, as seen in P58 (Fig. 4C and data not shown). Taken together, these data demonstrate that cells from P58 have a selective decrease of the proapoptotic protein BIM, which seems critical for cytokine withdrawal-induced apoptosis.

**P58 Has a Gain-of-Function NRAS Mutation**

To uncover the genetic basis of the BIM defect in P58, we sequenced the genomic locus of BIM and some of its regulators, including FOXO3a, FOXO1, FOXO4, ERK1/2, and JNK1/2, but found no mutations. We then carried out an unbiased screen by interrogating mRNA expression patterns in healthy controls and P58 lymphocytes at 0 and 24 h after IL-2 withdrawal by using microarrays. Two hundred and five probe sets were differentially expressed in P58 compared with controls (Fig. 5A; the microarray dataset is deposited in the GEO public database). Surprisingly, the gene expression pattern in P58 indicated the possible constitutive activation of the small GTPase, neuroblastoma RAS (NRAS) (Fig. 5B). This included up-regulation of two dual-specificity phosphatases (DUSP4 and -6) that are inhibitors of ERK signaling and were previously shown to be overexpressed in tumor cell lines containing somatic activating mutations in NRAS.
Figure 4. BIM down-regulation in P58 lymphocytes. (A) Analysis by immunoblotting of BIM and other BCL-2 members expression after IL-2 withdrawal (CW) in PBLs from a NL, P58, and an ALPS 1A patient. The isoforms extra-long (EL), long (L), and short (S) are indicated. β-Actin is a loading control. (B) Resting ex vivo PBLs and purified T and B cells from normals (NL) and P58 were lysed and assessed for BIM expression by immunoblotting. (C) Activated human lymphocytes were transfected with either nonsilencing RNAi (nsRNAi) or with a small interfering oligonucleotide directed at BIM (BIM RNAi) for 3 days, and then deprived of IL-2 or treated with anti-Fas antibody. Silencing efficiency was assessed by immunoblotting (Lower Right) for the nonspecific (ns) and the silencing (si) transfections. Data shown are the representative of three independent experiments. Shown is mean ± SD.

We examined the nucleotide sequence of NRAS in P58 and found a single heterozygous G-to-A transition causing a nonconservative aspartic acid substitution for glycine at codon 13 (G13D) (Fig. 5C). To exclude the possibility that this mutation was a
somatic event, we analyzed genomic DNA from various cells and tissues including lymphoblasts, unseparated resting peripheral blood mononuclear cells, purified monocytes, EBV-transformed B cells generated years before the diagnosis, as well as buccal epithelial cells (Fig. 5C and data not shown). The mutation was found in all hematopoietic samples but not in the epithelial cells, suggesting a somatic event affecting only the hematopoietic compartment. Accordingly, neither of the patient's parents, his sibling, or his two children harbored the mutant allele (data not shown). Importantly, the very same amino acid change in NRAS arises somatically in human pediatric and adult myeloid and lymphoid malignancies, but was never before documented in nonmalignant cells or tissues.

Mutations in codons 12, 13, and 61 are known to stabilize RAS proteins in an active, GTP-bound state by reducing intrinsic GTPase activity and causing resistance to GTPase-activating proteins. Consistent with the genetic alteration, we found that active NRAS was increased in P58 lymphocytes in serum-rich and, especially, in serum-poor conditions (Fig. 5D). We therefore hypothesized that the “gain-of-function” NRAS mutation may result in hyperactivation of the RAS/RAF/ERK pathway, which can negatively regulate BIM expression.

Hyperactive NRAS Induces BIM Down-Regulation Through ERK in Human Lymphocytes

To investigate the relationship between hyperactive NRAS and BIM suppression, we transfected human lymphocytes with wild-type (NRAS<sup>WT</sup>) or mutant NRAS (NRAS<sup>G13D</sup>). Overexpression of NRAS<sup>WT</sup> and especially NRAS<sup>G13D</sup> reduced BIM in H-9 cells and primary normal human lymphocytes (Fig. 6A and B), reminiscent of HRAS overexpression in epithelial cells. We consistently observed a 3- to 4-fold reduction in BIM, although this could be an underestimate of the effect because the transfection efficiency did not reach 100% in all cases.
Figure 5. Identification of a de novo gain-of-function NRAS mutation in P58. (A) Heat map and relational dendrogram results of a microarray study demonstrating the 205 probe sets differentially expressed by cells from P58 compared with cells from two normal subjects, NL1 and NL2. Activated lymphocytes were lysed at 0 and 24 h after IL-2 withdrawal, and mRNA expression was analyzed by using microarrays (B) Diagram demonstrating differential expression in P58 cells of genes in the NRAS/RAF/ERK pathway. Red and green arrows indicate the genes up- or down-regulated in P58, respectively, compared with controls. (C) Sequencing of NRAS by using genomic DNA from P58 lymphoblasts and monocytes demonstrating the heterozygous G-to-A, G13D substitution. (D) Active GTP-bound NRAS was immunoprecipitated before and after serum withdrawal in a NL and P58, by using a Raf-1 (RBD)-GST fusion protein as bait. The total quantity of NRAS in cell lysates before immunoprecipitation is also shown.
The most widely studied RAS effector proteins are either in the RAF/MEK/ERK pathway or the phosphatidylinositol 3-kinase pathway\(^1,2\). We found that chemical inhibition of MEK1, the MAP kinase kinase immediately upstream of ERK1/2, by using the drugs PD98059 or U0126 rescued BIM protein expression in P58 lymphocytes and NRAS-overexpressing H9 cells (Fig. 6C and data not shown). Remarkably, ERK inhibition also restored apoptosis after IL-2 withdrawal in P58 lymphocytes (Fig. 6D). Despite the strong increase in BIM protein levels after MEK inhibition, BIM mRNA varied little (Fig. 7A), consistent with a posttranscriptional, rather than transcriptional,
down-regulation of BIM by ERK. This was also true when resting lymphocytes from P58 were treated with MEK inhibitors in IL-2-rich conditions, and BIM protein and mRNA levels were measured (Fig. 7B and C). By contrast, the inhibitors LY294002 and wortmannin had no effect on BIM levels in P58 lymphocytes indicating that the phosphatidylinositol 3-kinase pathway was not involved (Fig. 7D and data not shown). Thus, active NRAS down-regulates BIM through the induction of the RAF/MEK/ERK pathway.

**Correction of the Apoptotic Defect by Farnesylation Inhibition and NRAS Silencing**

To further demonstrate the causative role of active NRAS in decreased BIM and defective lymphokine withdrawal apoptosis, we used two approaches. First, we used farnesyltransferase inhibitors (FTIs) that block the membrane localization and function of RAS proteins and are currently under clinical investigation for the treatment of human cancers\(^3\). Treatment with one such pharmaceutical, FTI-277, corrected the apoptotic defect and increased BIM levels in P58 lymphocytes (Fig. 8A). This drug had a negligible effect on apoptosis and BIM levels in normal cells (Fig. 8A).

Second, we decreased NRAS expression in P58 lymphocytes by using siRNA. Each of three different siRNA oligonucleotides reduced NRAS expression and restored BIM levels and sensitivity to apoptosis in P58 cells (Fig. 8B and C). By contrast, NRAS siRNAs had no significant effect on BIM or sensitivity to IL-2 withdrawal in normal cells (data not shown). These results verify that the heterozygous mutation causes an abnormal gain-of-function that is reversed by silencing or functional inactivation of NRAS.
Figure 7. Effects of MEK1 and PI3K manipulation in P58 cells. (A) Activated lymphocytes from two NL and from P58 were deprived of IL-2 and treated with either the DMSO vehicle or MEK1 inhibitors PD98059 (PD) (20 mM) or U0126 (10 mM) for 18 h, after which expression of BIM mRNA was measured by qPCR. (B) Samples from a NL and P58 were maintained in IL-2-rich media and treated with either DMSO or PD98059 for 18 h, and BIM expression was assessed by immunoblotting. (C) Quantitative real-time PCR analysis of BIM mRNA in a NL and P58 from the experiment described in (B). Results are representative of three experiments. Shown is mean ±SD. (D) Activated lymphocytes from a NL, an ALPS 1A patient and P58 were cultured under the described conditions in the presence or absence of the PI3-K inhibitor LY294002. BIM, BCL-2 and p27kip1 expression were analyzed by immunoblotting. The upregulation of p27kip1 expression upon cytokine withdrawal and in response to PI3-K inhibition served as a positive control for the treatments.
Figure 8. Correction of the apoptotic defect in P58 by inhibition of farnesylation and RNA silencing. (A) Activated lymphocytes from P58 and a normal control (NL) were deprived of IL-2 and treated daily with DMSO or FTI-277 (5 µM). (Inset) Resting lymphocytes from P58 were treated with DMSO (−) or 2, 5, or 10 µM FTI-277, and BIM expression was analyzed by immunoblotting. (B) Activated lymphocytes from a NL and P58 were transfected with nonsilencing (nsRNAi) or three different NRAS-targeted siRNA oligonucleotides (NRAS#1/2/3), and BIM expression was measured 3 days later by immunoblotting. (C) Activated lymphocytes from a NL and P58 were transfected as described in B and subjected to IL-2 withdrawal, and apoptosis was measured daily for the indicated period of time. Data shown are representative of three or more independent experiments. Shown is mean ± SD.
NRAS was identified as a transforming factor in neuroblastoma and other malignancies, but its principal physiological role in humans has been uncertain. Our genetic evidence suggests that an activating somatic NRAS mutation causes BIM down-regulation and defective intrinsic mitochondrial apoptosis prominently in lymphocytes, leading to the key features resembling ALPS and hematopoietic malignances.

Given the somatic origin of the NRAS mutation in our patient, the clinical phenotype contrasts with the previously known human genetic disorders due to mutations in p21 RAS oncoprotein pathways, including Costello syndrome (HRAS), Noonan syndrome (PTPN11, KRAS, SOS1) and cardiofaciocutaneous syndrome (MEK1, MEK2, B-RAF, and KRAS) that comprise developmental aberrations and neoplastic transformation of various mesodermal and ectodermal tissues. Transgenic mice bearing activating NRAS mutations develop several hematological tumors, such as leukemia, lymphoma, mastocytosis, and rare mammary carcinomas.

Our observations reveal a vital role of intrinsic mitochondrial apoptosis in peripheral lymphocyte homeostasis and tolerance in humans. Although gene manipulation in rodents suggested that this pathway was important for peripheral immune homeostasis, our human data validate the hypothesis with some surprising twists. The NRAS mutation caused a clinical phenotype that, in several important aspects, is similar to other ALPS patients, with modestly elevated TCR-αβ+CD4-CD8- T cells, chronic lymphoid accumulation, and a clear propensity to hematological tumors. However, certain other immunophenotypic and histological features of ALPS were not seen such as marked expansions of DNTs in the lymph nodes and elevated HLA-DR+ T cells and CD57+ T cells in the periphery. The apoptosis defect is also clearly different from all previous ALPS cases and underscores a confusing point in the literature. Originally, the ALPS phenotype was based on defects in TCR-induced apoptosis indicating a deficiency of the propriocidal regulatory mechanism. Because many cases over the years were identified with mutations in the Fas receptor, it was generally assumed that ALPS could be defined by a defect in Fas-induced apoptosis. If we adopt a broader view of apoptosis defects that could disturb peripheral lymphocyte homeostasis,
then P58 reveals another derangement of this regulatory process. Because we favor this view, it is reasonable to consider P58's lymphocyte apoptosis defect to fulfill one of the diagnostic criteria of ALPS. Because he also exhibits increased αβDNTs and expanded secondary lymphoid tissue, he would manifest all of the required features for a diagnosis of ALPS. Because his overall phenotype and genotype are distinctive, and clearly different from ALPS types I and II, we provisionally name this condition ALPS, type IV (type III represents undefined molecular pathogenesis).

The active NRAS phenotype we observed is different from homozygous deficiency of BIM in rodents, because the latter does not cause an increase in CD4^-CD8^-αβ T cells, and induces the expansion of other cell types, such as granulocytes. These differences may reflect either the residual expression of BIM or the stimulatory effects of NRAS on ERK and other downstream RAS effectors, which could have direct mitogenic effects that are not triggered by BIM modulation. Moreover, there are several regulatory levels between NRAS and BIM where other factors could account for the difference between the BIM knockout mice and P58.

The mechanism of BIM suppression under conditions of hyperactive NRAS is not clear. In other cellular models of hyperactive KRAS, BIM is down-regulated through phosphorylation, ubiquitination, and degradation by the proteasomal machinery. Here, we show that BIM protein levels are severely depressed in resting and activated T cells from P58 compared with normals, despite equivalent basal mRNA levels. Additionally, the usual up-regulation of BIM mRNA after IL-2 withdrawal was impaired in cells from P58. Also, BIM protein returned to almost normal levels on MEK/ERK blockage, but mRNA remained the same. In our preliminary experiments (data not shown), there was no change in BIM levels on proteasomal blockade in lymphocytes from P58. Taken together, these data suggest a double mechanism for BIM suppression via hyperactive NRAS: inhibition of up-regulation of mRNA and translational inhibition, but this remains to be formally shown. Our new understanding of NRAS suggests that RAS antagonists such as FTIs could have beneficial effects on disorders of lymphocyte homeostasis and autoimmunity in addition to cancer.

References
NRAS in an ALPS-like syndrome


